Int. J. Plant Sci. 170(4):444–456. 2009.

1058-5893/2009/17004-0002$15.00 DOI: 10.1086/597273

MULTICELLULAR SECRETORY TRICHOME DEVELOPMENT ON SOYBEAN AND RELATED *GLYCINE* GYNOECIA

R. A. Healy,* R. G. Palmer,† and H. T. Horner[1,]*

*Department of Genetics, Development and Cell Biology and Microscopy and NanoImaging Facility, Iowa State University, Ames, Iowa 50011-1020, U.S.A; and †Department of Agronomy and USDA-ARS Corn Insects and Crop Genetics Research (CICGR) Unit, Iowa State University, Ames, Iowa 50011-1010, U.S.A.

Multicellular glandular trichomes form on gynoecia of wild annual *Glycine* species, annual soybean cultivars, and wild perennial *Glycine* species. These trichomes occur from the ovary base to the style base and, in perennial species, along the style as well. Trichomes form at least 2 d before anthesis, and new trichomes develop throughout flowering and also on young seed pods. Trichome structure is similar in all taxa examined, usually five to seven linearly arranged cells. Stalk cells with callose walls become highly vacuolate, and their cytoplasms have reduced numbers of Golgi bodies and endoplasmic reticulum. During secretion, two to four distal cells develop dense cytoplasms containing both prominent Golgi bodies with large vesicles and endoplasmic reticulum with enlarged lumens. Both organelles are involved in forming carbohydrate/protein secretory products. Internal secretion begins with products exuding between the plasmalemma and the primary wall of each distal cell. Secretion progresses between the primary wall and the outermost cuticle that entirely covers the trichome, which is thicker and more highly modified than a normal cuticle. This cuticle separates from cell walls in secretion regions. Products are exuded outside, occur on the inner surface of trichome and gynoecium, and are more obvious in perennial than in annual taxa. The composition and function of products are unknown.

Keywords: annual, cultivar, *Glycine*, gynoecia, Leguminosae, soybean, trichomes, wild annual, wild perennial.

Introduction

Surface hairs, or trichomes, are a common feature of the epidermis of many plant organs and may serve a variety of functions, including mechanical and chemical protection against herbivores, production of cloth and other useful materials, and synthesis of medically, pharmacologically, or commercially important compounds (Esau 1965; Lüttge 1971; Fahn 2000; Werker 2000; Wagner et al. 2004). Some of these functions are being enhanced by use of both genetic and molecular approaches (Payne et al. 2000; Kim and Triplett 2001; Wang et al. 2001, 2004; Wagner et al. 2004). Unicellular and multicellular trichomes are found in a variety of shapes and sizes and may or may not be secretory (Fahn 2000). The literature is replete with studies of trichomes from throughout the plant kingdom, demonstrating both their variety and their importance to their plant systems. Elucidation of how they form (Szymanski et al. 2000), are spaced on organs (Larkin et al. 1996), and develop and carry out their functional role(s) has opened a broad area of research, particularly for crop plants, where trichomes may participate in unique processes contributing to plant health, quality, and yield (Wagner et al. 2004). Included in this list are the production of compounds that entrap or repel insects and pathogenic microorganisms or attract insect pollinators (Levin 1973; Eisner et al. 1998; Wang et al. 2001; Carter et al. 2007; Horner et al. 2007; Junior et al. 2008).

[1] Author for correspondence; e-mail: hth@iastate.edu.

Manuscript received August 2008; revised manuscript received November 2008.

Plants whose organs may have no trichomes are called glabrous, while other species may be pubescent, with few to many trichomes; plants in the latter condition are termed "hirsute." There may be one type of trichome or several types, depending on location on the plant and the stage of organ development. The genus *Glycine* (Leguminosae) is a good example of the latter condition. Legumes and other nonleguminous taxa display a variety of uni- and multicellular trichomes, both nonsecretory and secretory, on the vegetative (Akers et al. 1978; Franceschi and Giaquinta 1983; Retallack and Willson 1988) and floral organs (Guard 1931; Bernard and Singh 1969; French 1987; Gunasinghe et al. 1988; Healy et al. 2005). Specifically, a diploid cultivar (cv) of soybean called Clark has eight near-isogenic lines—glabrous, puberulent, sparse, sharp hair tip, dense 1, dense 2, extra dense, and normal—that all display a variety of trichome conditions associated with the gynoecia. These eight genotypes were studied in detail to determine the frequency, location, and types of trichomes associated with the gynoecia, as part of another study dealing with the floral nectary that circles the base of the gynoecium (Horner et al. 2003).

Healy et al. (2005) described three types of soybean trichomes associated with the gynoecia: unicellular, thin walled, and nonsecretory (UCT); multicellular, thick walled, and nonsecretory (TWT); and multicellular and secretory (MCT). Franceschi and Giaquinta (1983) described MCTs only on soybean leaves, but their report did not include any mention of the other two types of trichomes. Further characterization of the *Glycine* gynoecial MCTs is important because evidence will show that it secretes visible products in close proximity to floral nectaries, suggesting a potential for a novel role in nectar production or

other undefined processes. No such product was observed in association with the leaf MCTs (Franceschi and Giaquinta 1983). The increase in studies dealing with floral nectaries, nectar, and its milieu of components (Nicolson and Thornburg 2007) raises questions as to whether the *Glycine* gynoecial MCTs actually form secretory products; whether these products can be visualized and chemically identified; and, if so, what possible function(s) these products have in the gynoecium-nectary-flower complex. These questions are significant, particularly for soybean, a plant that is capable of attracting insects for cross pollination and hybridization but does so in a very limited way (Palmer et al. 2001).

This study greatly extends the earlier research by Franceschi and Giaquinta (1983) and provides new insights for future studies that could positively affect development of hybrid soybean. Ongoing developmental and molecular research on ornamental tobacco nectaries (Thornburg et al. 2003; Carter et al. 2007; Horner et al. 2007; Ren et al. 2007*a*, 2007*b*) and soybean (Horner et al. 2003; Carter et al. 2007) are beginning to provide some interesting insights to these issues.

Material and Methods

Plant Material

Gynoecia (fig. 1*A*) were dissected from flowers (fig. 1*B*) of wild perennial *Glycine tomentella*, both diploid (G 1300 [PI 441.000]) and tetraploid (G 1133 [PI 441.001]); wild annual *G. soja* Siebold. & Zucc.; and two annual *G. max* (L.) Merr. (soybean) cultivars, Raiden (PI 360.844) and Clark (PI 548.533). Unless otherwise noted, all *G. tomentella* examples in text and figures are diploid. Plants were grown either in the USDA greenhouse or at the Bruner Farm near Ames, Iowa. Flowers were harvested between 9:00 and 11:00 a.m. to standardize comparisons. Field-grown cultivars of *G. max* were harvested in August 2004. Flowers from greenhouse-grown Clark were harvested and fixed in July 2004, and Raiden was harvested and fixed in March 2005. Flowers from greenhouse-grown *G. soja* and *G. tomentella* (diploid) were harvested and fixed when the plants flowered profusely in January 2005. Even though the time of flowering differed between the field-grown and greenhouse-grown plants, the developmental sequences of gynoecia and trichomes were the same. Gynoecia were collected at three stages of development: 2 d before anthesis (dpa), 1 dpa, and at anthesis, the day of flower opening. Flowers were staged according to accepted procedures for soybean (Fehr et al. 1971).

Processing Gynoecia for Microscopic Observations

Gynoecia were dissected from the flowers at the three stages identified above and immediately immersed for 5 h in fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) at room temperature (21°C) under a low vacuum (18 psi) and then stored in the buffered fixative overnight at 4°C (see Healy et al. 2005). The gynoecia then were dehydrated through an ethanol series and embedded in LR White resin (London resin white) for general and histochemical staining for LM. Sections 1 μm thick were cut on a Reichert Ultracut S microtome (http://www.leica-microsystems.com) with glass knives and placed on poly-L-lysine-coated glass slides for staining.

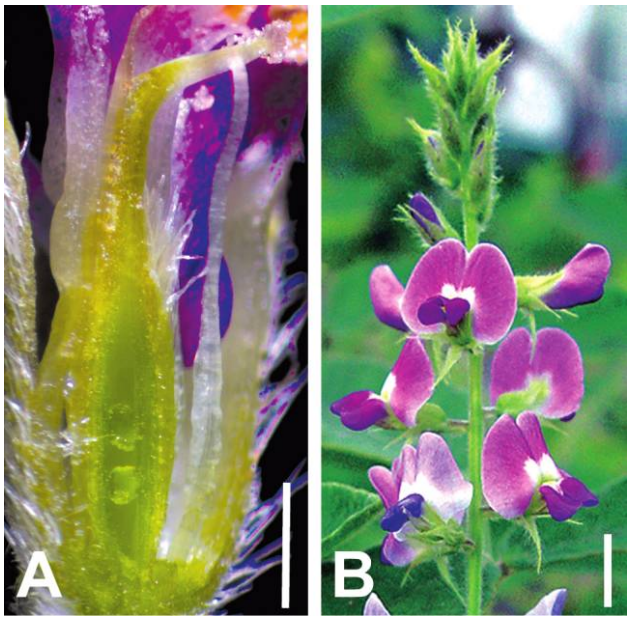

Fig. 1 Macrophotographs of *Glycine* gynoecium and flowers. *A*, Dissected flower (*G. max* cv. Clark) showing portions of calyx, stamens, and central gynoecium with stigma and style. *B*, Flowering stem (*G. tomentella*, tetraploid) with flowers at different stages of maturity. Note that these organs are covered with many visible, elongated trichomes. Scale bars = 0.5 mm in *A*; 5 mm in *B*.

Gynoecia prepared for SEM and TEM were initially fixed in the same way as those for LM, with the addition of postfixation with 1% osmium tetroxide in the same buffer for 1.5 h at 21°C, then washed in buffer and dehydrated through 100% ethanol. The gynoecia used for SEM were critical-point dried, mounted on aluminum stubs with double-sided adhesive pads, and coated with gold/palladium (10 nm thick; Denton Sputter Coater; http://www.dentonvacuum.com). The gynoecia used for TEM were transitioned from 100% ethanol to pure acetone and embedded in Spurr's resin (Spurr 1969 [hard mixture]; Healy et al. 2005). Ultrathin sections (60–80 nm) were cut with a Diatome diamond knife (http://www.diatome.ch) and placed on Formvar-coated slotted copper grids. The sections were poststained with lead citrate and ethanolic uranyl acetate.

As defined by Healy et al. (2005; their fig. 2*A*), the gynoecium, from the confluence of its base with the base of the surrounding nectary to the base of the stigma, is divided into five zones to aid in depicting locations of the gynoecial trichomes. Zone 1 begins at the base of the gynoecium and ends at the top of the nectary; zone 2 begins at the top of the nectary and extends to the top of the second ovule (usually three ovules are present) or the top of the first ovule when only two ovules are present (since *G. tomentella* usually has eight ovules, zone 2 in that species extends to the top of the fourth ovule); zone 3 begins at the top of the second ovule, or the top of the first ovule when only two ovules are present, and ends where the style begins; zone 4 consists of the straight portion of the style up to where it begins to curve; and zone 5 includes the curved portion of the style up to the base of the stigma. Almost all of the MCTs from the five taxa used in

this study were located in zones 2 and 3 (which cover the major part of the ovary).

Quantification of Secreting MCTs

MCTs were counted on gynoecia of *G. tomentella* and Raiden and compared. Longitudinal sections of 25 resin-embedded, PAS-stained gynoecia collected at anthesis from each species were viewed with bright-field microscopy with a 60× objective. Counts included all MCTs, subdivided into mature MCTs and MCTs with evidence of secretion onto the gynoecium surface in zones 1–4. Maturity was determined by the appearance of secretory product in out-pocketing of apical cells (or collapse of these out-pockets) and constricted, deeply staining cytoplasm in apical cells. The counts were repeated with a different section on each slide, and the results were averaged.

Contrast Enhancement and Histochemistry

General contrast enhancement of LM resin sections was done by using toluidine blue O, a metachromatic dye that differentially stains the cellular substrates. Localization of callose (β-1,3 glucans) was identified in 5-μm-thick deparaffinized sections by using 0.005% aniline blue in 0.15 M K_2PO_4 at pH 8.2 (Currier 1957). The aniline blue solution was placed on the sections, a coverslip was added, and sections were viewed with a Zeiss Axioplan II compound microscope (http://www.zeiss.com) in reflected-fluorescence mode with a UV excitation filter (265 nm) and a barrier filter cutoff at 430 nm. All images were digitally recorded with Zeiss MRc color cameras mounted on a Zeiss Axioplan II (fluorescence) or an Olympus BH40 (bright field; http://www.olympus.com) compound microscope.

Histochemistry was used to localize proteins (amido black 10B stain; Wilson 1992) and non-water-soluble polysaccharides (α- and β-1,4-linked polysaccharides; PAS stain; Ruzin 1999) with LM. Sections for TEM (Jeol 1200 EX; http://www.jeol.com) were stained according to Healy et al. (2005) and observed at 80 kV. The SEM (Jeol 5800 LV) was operated at 10 and 15 kV and a working distance of 15 mm. SEM specimens were mounted on aluminum stubs with double-stick adhesive tabs and silver paint and then coated with gold/palladium. All digital images captured by LM, SEM, and TEM were processed with Adobe PhotoShop and Adobe Illustrator CS3 software (http://www.adobe.com).

Results

MCTs have their origin in the epidermis of both vegetative (leaves [Franceschi and Giaquinta 1983] and stems) and floral organs, such as gynoecia (fig. 2*A*; Horner et al. 2003; Healy et al. 2005; this study). Many gynoecial epidermal cells divide to form stomata and one of three types of trichomes: UCT, TWT, and MCT. These three types of trichomes (fig. 2*B*) are intermingled on the surface of the gynoecia from the various taxa and display different frequencies, depending on their position in the five zones of the gynoecia from base to style (Healy et al. 2005). The MCTs are most frequent in zones 2 and 3, which include the main part of the ovary wall (Healy et al. 2005). Images of the developmental stages (2 dpa, 1 dpa, anthesis) in this section accurately represent all these taxa, which have highly similar MCT structures.

Initiation of MCTs (2 dpa)

The earliest MCTs are initiated at least 2 dpa on the immature gynoecia. Some epidermal cells in zones 2 and 3 expand and produce protrusions with vacuoles located proximally and dense cytoplasm distally (fig. 2*C*). The first periclinal division forms two cells: a stalk cell with a larger vacuole and a distal cell with denser cytoplasm and, typically, smaller vacuoles (fig. 2*D*, 2*F*). MCT initiation appears to take place continuously as the gynoecium enlarges. Thus, a series of MCTs are produced at different stages of development during the 3-d life (2 dpa, 1 dpa, and anthesis) of the gynoecium, before fertilization, and before pod development.

Development of MCT Stalk and Distal Cells

After the first two cells are formed, additional periclinal divisions (fig. 2*E*, 2*G*) increase the number of linearly arranged cells to, typically, five to seven (fig. 3*B*, 3*E*). However, up to 10 cells have been observed for a single trichome (Clark sparse near-isogenic line; Healy et al. 2005). Periclinal divisions generate highly vacuolated stalk cells, but the distal cells contain many small vacuoles that surround each prominent central nucleus, and they have noticeably denser cytoplasm (figs. 2*G*, 3*A*).

MCT Stalk Cells

The stalks of *Glycine tomentella* (and the other taxa in this study) typically consist of the original epidermal cell and two or three more cells that we term "stalk" cells. All these cells are highly vacuolated, with each displaying a nucleus that is initially similar in size and density to those of the most distal cells (fig. 3*A*, 3*B*). The stalk-cell nuclei retain this appearance throughout MCT development, and later in development they are generally found to one side of each vacuolated cell. The external morphology of the MCT shows a series of robust linear cells separated by periclinal cross walls (indented rings; fig. 3*C*).

Primary cell walls forming the boundaries of stalk cells and covering more distal MCT cells stain magenta for non-water-soluble polysaccharides (fig. 3*D*, 3*E*). The cross walls of the linearly arranged MCT also stain magenta, denoting that they contain non-water-soluble polysaccharides, including those that form partitioning walls (fig. 3*D*, small arrow). Not stained is a layer to the outside of the primary walls (fig. 3*D*, 3*E*, arrowheads). In electron microscopy, the cuticle covering the nontrichome gynoecium (fig. 3*F*, arrowheads) is electron dense, whereas the modified cuticle on the MCT is larger and stains much less intensely (fig. 3*F*, asterisk). The juncture of these two cuticles is obvious (fig. 3*F*, arrows).

The internal cross walls that separate the linearly arranged stalk cells are thinner and are contiguous with the outer primary walls (fig. 4*A*), and the partitioning walls contain distinct plasmodesmata (fig. 4*B*, 4*C*). The PAS staining of these partitioning walls is less intense than that of their outer primary walls later in MCT development, which suggests a difference in structure and chemical composition (see "Stalk Cell Wall Callose").

Even though the cytoplasm of the stalk cells is more vacuolate than that of the distal cells, each cell contains a normal

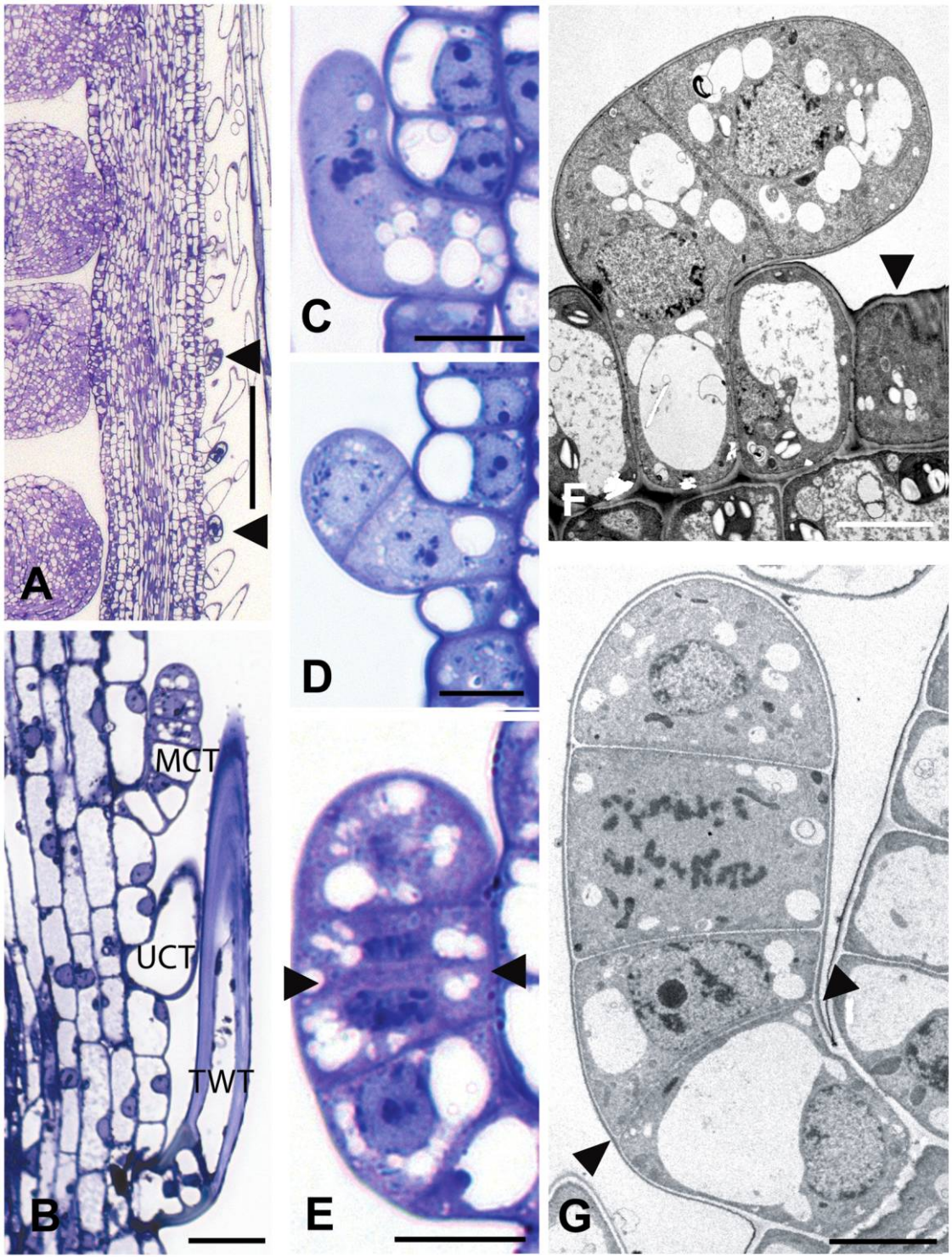

Fig. 2 Portion of *Glycine* gynoecium and epidermis with early stages of developing trichomes, observed with LM (toluidine blue O stained) and TEM. *A*, Portion of gynoecium (*G. tomentella*) in zones 2 and 3 showing mixture of trichomes (arrowheads); all trichomes oriented toward style and stigma. *B*, Magnified portion of gynoecium epidermis (*G. soja*) in zone 2 displaying three types of developing trichomes (*MCT*, *UCT*, and *TWT*). *C*, Elongated MCT initial cell (*G. tomentella*) before first cell division. Note large basal vacuoles and bend of cell toward style. *D*, Two-celled MCT (*G. tomentella*) with obvious cross wall. *E*, Four-celled MCT (*G. tomentella*) with proximal terminal cell in division (arrowheads). Basal cells have larger vacuoles than terminal cells. *F*, Two-celled MCT (*G. max* cv. Raiden; comparable to *D*) showing vacuolated cytoplasms, a single cross wall, and an outer wall with a lightly stained modified cuticle layer covering the MCT that is different from the densely stained cuticle layer (arrowhead) over adjacent epidermal cells. *G*, Four- to five-celled MCT (*G. tomentella*) in division (comparable to *E*) showing a difference in appearance between the lowermost cross wall (arrowheads) and the two uppermost cross walls. Outer MCT modified cuticle layer is less dense than cuticle on epidermal cells. Scale bars = 150 μm in *A*; 20 μm in *B*; 10 μm in *C–E*; 5 μm in *F*, *G*.

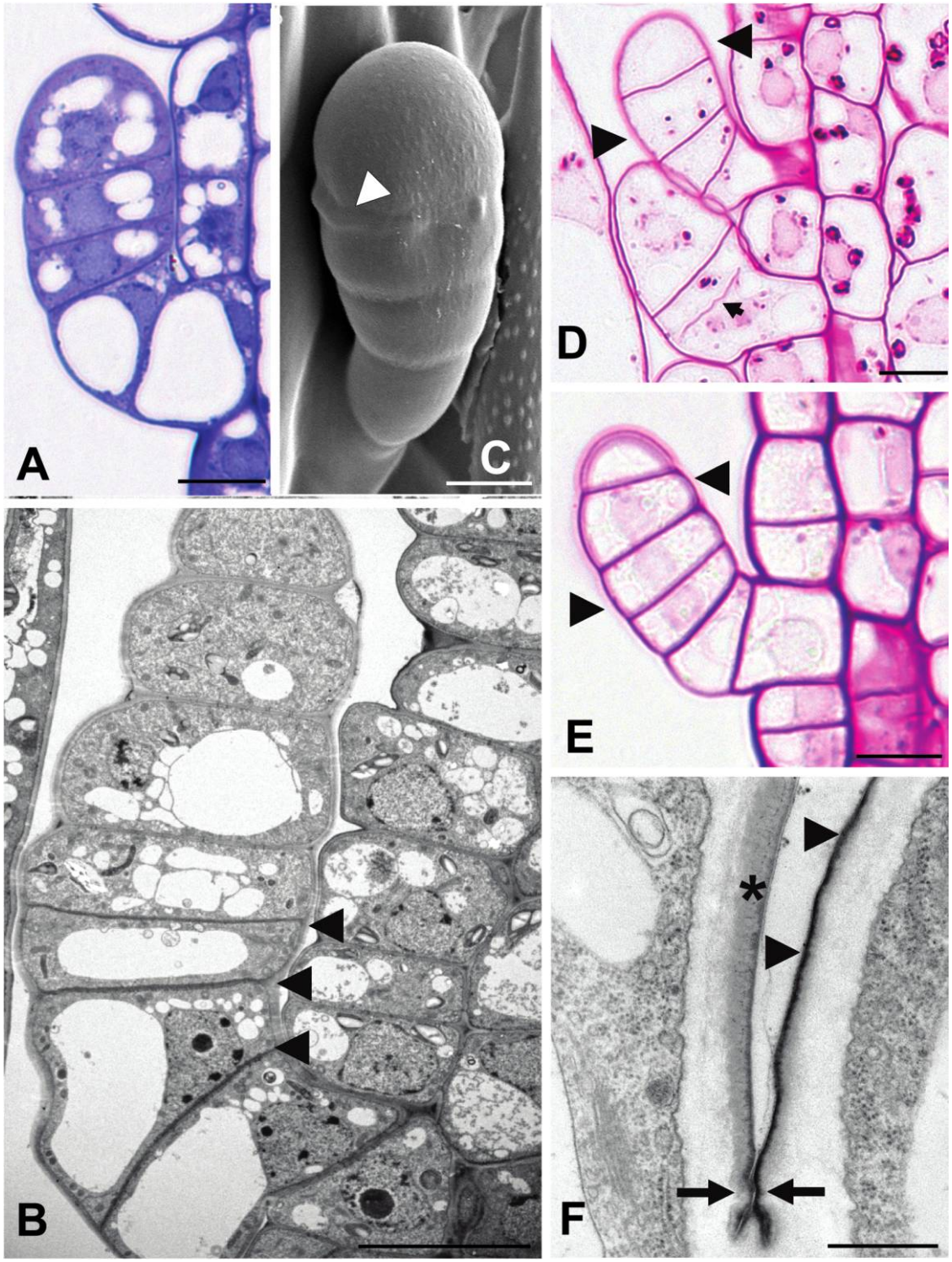

Fig. 3 Stages of *Glycine* MCT development that focus on cell wall development, viewed with LM, SEM, and TEM. *A*, Five-celled MCT (*G. tomentella*) showing differentiation between highly vacuolated stalk cells and terminal cells (toluidine blue O staining). *B*, Eight-celled MCT (*G. max* cv. Raiden) with basal (stalk) cross walls (arrowheads) more electron dense than terminal cross walls. *C*, Five-celled MCT (*G. tomentella*) with a slight bulge near uppermost cross wall (arrowhead) toward gynoecium. *D*, Young, PAS-stained MCTs (*G. tomentella*) showing starch grains and intensely stained primary outer and cross walls, one cell plate in lower basal portion of MCT (small arrow), and thin, unstained outer modified cuticle (large arrowheads) covering entire portion of MCT. *E*, Slightly older MCT (Raiden) with intensely stained outer and cross walls and prominent modified cuticular layer (arrowheads). *F*, Juncture (arrows; *G. tomentella*) between basal cell wall of MCT (left) and epidermal cell wall (right). Thin, electron-dense cuticle occurs on surface of epidermal cell wall (arrowheads), whereas the modified MCT cuticle layer (asterisk) is much thicker and much less electron dense. Scale bars = 10 μm in *A–E*; 500 nm in *F*.

complement of organelles. Conspicuous strands of rough endoplasmic reticulum (RER) and conspicuous Golgi bodies have vesicles containing electron-translucent contents. These vesicles are near or attached to the plasmalemma before and during callose deposition (see "Distal Cells: Secretion (Anthesis)").

Stalk Cell Wall Callose

High-pH aniline blue staining of the MCTs from all taxa studied indicates the presence of callose, a β-1,3 glucan polymer. Fluorescence microscopy clearly shows that both outer and partitioning walls of the stalk cells fluoresce with variable brightness (fig. 4*D*, 4*E*). The MCTs of all taxa stained in this manner displayed the presence of callose. Callose-staining intensity is strongest in the peripheral stalk cell walls but varies in intensity in the partitioning walls containing the distinct plasmodesmata. Differential staining of the cell walls shown in figure 4*D*, 4*E* is due to differences in developmental age of the individual MCTs.

Distal Cells: Presecretion Products (1 dpa)

The two or three distal MCT cells, once formed, become visually different from the basal stalk cells. Their nuclei are slightly larger, stain more densely, and are surrounded by many small vacuoles (fig. 4*F*, 4*G*). In addition, their cytoplasms become increasingly dense, displaying more ribosomes, arrays of RER, extensive Golgi apparati with many vesicles, mitochondria, plastids, and small vacuoles (fig. 4*H*) than the basal stalk cells. These dense cytoplasms appear to be "presecretory factories" for the next phase of development (next section). Vesicles occur in the cytoplasm and are attached to the plasmalemma at this time. The other primary cell wall and the modified cuticle layer appear slightly larger and less dense (fig. 5*A*). Secretory products build up between the plasmalemma and the outer primary wall, exerting pressure on cellular contents, which become more compact. Some products are seen between the primary wall and the outer modified cuticle and cause the cuticle to bulge preferentially toward the epidermis of the gynoecium (fig. 4*F*, 4*G*).

Distal Cells: Secretion (Anthesis)

The dense cytoplasm of many MCT distal cells displays Golgi apparati, consisting of Golgi bodies and associated vesicles, and RER (fig. 5*B*, 5*C*). The vesicles associated with the Golgi bodies contain an electron-translucent product. The disassociated vesicles appear to fuse with the plasmalemma. Concomitantly, the extensive RER lumens enlarge to reveal fibrous material (fig. 5*C*, 5*D*). The RER strands have swollen regions, which sometimes are fused with the plasmalemma (fig. 5*C*).

Both the Golgi vesicles and the RER seem to secrete their materials (products) first between the plasmalemma and the primary cell wall, then between the lateral primary cell wall and the outer wall layer (modified cuticle), and finally to the outside, where the products are quite visible and abundant toward the gynoecium, especially in the wild perennial MCTs. This progression of secretory products is commonly evident between the most distal and the next two more proximal cells (fig. 4*F*; fig. 5*E*–5*H*). The densely staining and compact cytoplasm of these secreting distal cells, containing secretory machinery, is concentrated in the center of each cell. The secreted products build up between the plasmalemma and the primary cell wall faster than they can be transferred through the primary wall and the outermost modified cuticle layer. This further suggests that each MCT secretory period is relatively short and intense.

The vacuoles in distal cells also appear to be filled with a speckled, electron-translucent material (fig. 5*B*). No vesicles or RER strands were shown to be specifically associated with these vacuoles during the secretory phase. These vacuole contents are specific to the distal cells and contain lightly staining material that occurs at this stage and at 1 dpa (fig. 4*H*, asterisk). Besides the organelles already mentioned, the mitochondria are numerous and well developed. The plastids at this stage, which contain unstacked thylakoids for the most part, quite often contain crystalline bodies of unknown composition (fig. 5*B*, 5*D*).

The bulging of the modified cuticle layer toward (and sometimes to the opposite side of) the gynoecial epidermal cell layer is a common feature (fig. 4*F*, 4*G*) that was observed beginning at 1 dpa. The secretory products appear as a homogenous mass when stained with toluidine blue O, cover most of the MCT, and adhere to the gynoecial epidermis (fig. 5*E*, 5*F*, 5*H*).

Quantification of MCTs with Visible Secretory Products

There was an obvious difference between the abundance of MCTs that visibly displayed secretory products next to the gynoecia of *G. tomentella* and that of any of the annual species or cultivars. To quantify this observation, we chose Raiden for a comparison with *G. tomentella* of the numbers of secreting MCTs because these are the two species that we used to follow MCT development at the ultrastructural level. There were more than twice as many mature trichomes among MCTs on *G. tomentella* as on Raiden, and far more of the mature MCTs had visible secretory products on *G. tomentella* than on Raiden (52% vs. 4%; table 1).

Histochemistry of Secretory Cells and Their Products

General toluidine blue O binds differentially to almost every part of the MCT. This differential contrast implies that each MCT cell contains a variety of substrates. The secretory products, as mentioned above, stain a uniform light blue (fig. 5*E*).

Proteins. Amido black 10B staining for general proteins enhanced contrast in the cytoplasm of all MCT cells, especially the distal secretory cells (fig. 5*F*). The products between the primary walls and the modified cuticle of the distal cells also stained lightly, as did the products secreted to the outside (fig. 5*E*, asterisks).

Non-water-soluble polysaccharides. Two major substrates in the MCTs that are PAS positive are the cellular starch grains (fig. 5*H*) and the stalk and distal peripheral cell cross walls (fig. 5*G*, 5*H*). Depending on the taxon, the starch grains vary in number during the two days before anthesis (cf. figs. 3*D* and 3*E*) and are absent at anthesis, the time of nectar secretion. The basal walls surrounding all MCTs vary dramatically in their staining for non-water-soluble polysaccharides. In addition, the secretory products stain positively with the PAS technique between the distal primary walls and the modified cuticle (fig. 5*G*). These products are retained on the outside of the MCTs around

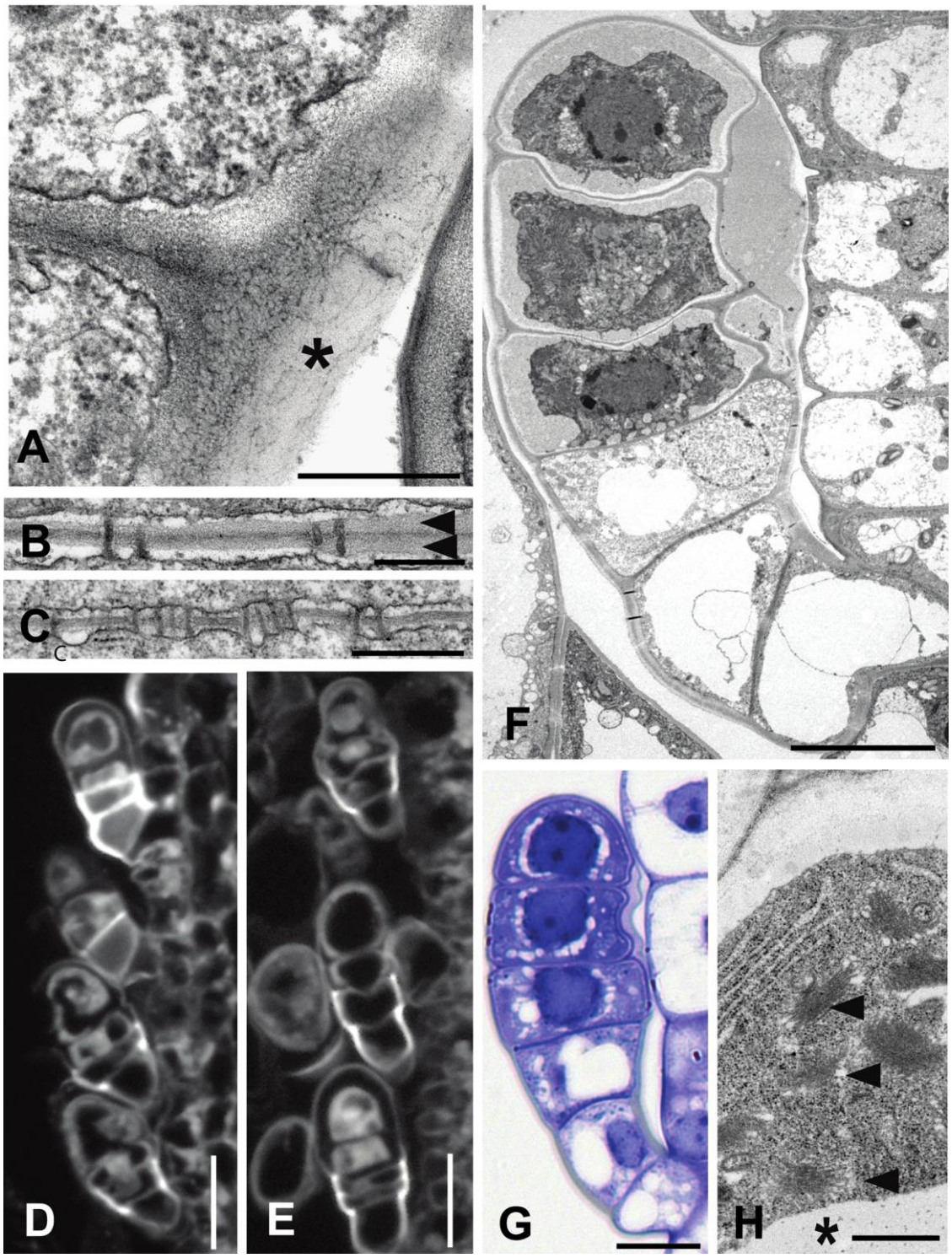

Fig. 4 Stages of *Glycine* MCT development viewed with LM and TEM. *A*, Portion of outer and cross walls (*G. max* cv. Raiden) showing altered primary walls and expanded, electron-translucent, modified cuticle layer (asterisk); compare epidermal cell wall to right. *B*, Portion of cross wall (Raiden) separating two terminal cells. Composite wall consists of middle lamella, two primary walls (arrowheads), outer electron-translucent layers, and traversing plasmodesmata. *C*, Portion of cross wall (Raiden) separating two basal stalk cells. This wall is thinner than terminal cross walls because primary walls are thinner. Portion of outer electron-translucent layers are considered to be callose. Relatively more plasmodesmata traverse these basal walls than the cross walls in *B*. *D*, Fluorescence image of MCTs (Raiden) along gynoecium with positively staining walls indicating the presence of callose. Some lowermost basal outer and cross walls contain callose. *E*, Fluorescence image of MCTs (Raiden), similar to *D*, showing outer basal cell walls differentially and positively stained for callose. *F*, Six-celled MCT (Raiden) displaying three

the distal cells, stain intensely (fig. 5*H*), and are conspicuous, particularly next to the gynoecium, as shown by SEM (fig. 5*I*).

In summary, the MCTs of the taxa studied are similar developmentally and structurally in their 2–3-d life before young pod formation. They have an intense but short period of internal and external secretory activity and form visible products containing unidentified glycoproteins. These products are secreted by the distal cells through primary walls and a modified cuticle toward the epidermal walls, typically in zones 2 and 3 of the gynoecium.

Discussion

Glandular trichomes are involved in an array of functions that equal the variety and complexity of the products they produce (Spring 2000). The number of studies dealing with the development of these trichomes is increasing (Wagner et al. 2004). This level of interest will continue, especially with respect to plants that are of economic importance. The annual soybean has at least three types of trichomes, including two relatively large nonsecretory trichomes and very short glandular trichomes. These trichomes occur on vegetative and floral aerial appendages, including the gynoecium in the center of the enclosed flower (Healy et al. 2005).

The outer gynoecial walls of wild perennial and wild and cultivated annual *Glycine* species are variously covered with these three types of trichomes, with the MCTs being the shortest and the only secretory type. Franceschi and Giaquinta (1983) found MCTs on the leaves of *G. max*, but their study did not demonstrate any secretory products and, as a result, provided no information about the metabolic machinery and mode of transport of such products to the outside of the trichome. It is possible that exposure on the leaves (and probably stems) may have caused the products to evaporate or be dissolved by water. Another possibility is that the more exposed MCTs do not produce an appreciable amount of products, if any. This could suggest a division of labor between the vegetatively formed MCTs and their counterparts, within the flower, associated with gynoecia.

Chemical Nature of MCT Secretory Products

This study focused on the MCTs that are surrounded by the flower appendages in a closed floral environment, preventing evaporation and/or water dissolution. Even though these MCTs are still present on the young seed pods, this study did not determine whether they continue to produce products. In the earlier stages of gynoecium development, the secretory products (or portions of them) were retained in all chemical preparations in our study. They are visible in LM, SEM, and TEM images. Until this study, there was no information about how the secretion products may be synthesized and transported to the outside of the trichome. It has been impossible to collect the products for compositional analysis because of the minute quantities produced by the MCTs, their very small size, and the frequency and much larger size of the nonsecretory trichomes covering them. However, the secretory products appear to be somewhat resistant to (and possibly only partially soluble by) aqueous fixatives and the ethanol and acetone dehydrating fluids. With the general histochemical procedures used in this study on fixed MCTs, the remaining products variably stain for a mixture of general proteins and α- and/or β-1,4-linked non-water-soluble polysaccharides. In that the ultrastructural machinery involved in their production and secretion consists of Golgi bodies, vesicles, and RER (with no spherical lipidlike bodies present, based on TEM fixation and imaging), we believe that the remaining products primarily consist of glycoproteins but not lipids, both of which are found in a variety of other types of glandular trichomes (Spring 2000).

It is not possible, without definitive chemical analyses, to ascribe a functional role to the products or to assume that the products are identical to those presumably produced by similar MCTs that occur on the vegetative structures, such as leaves and stems. Franceschi and Giaquinta (1983) suggested a potential role for the products in insect repellency. This is a possibility for the gynoecia-associated MCTs, as is deterrence of the microbes that certainly are within the floral cavity and thus in contact with the floral nectar. Such a function is possible, because in situ hybridization localization of *NOX1* expression has been shown for soybean nectaries and MCTs as well as tobacco nectaries (Carter et al. 2007). *NOX1* is an NADPH oxidase that produces superoxide that is toxic to microorganisms in floral nectar. Other authors also indicate the secretion of a toxic product from trichomes against herbivores (Thurston et al. 1966; Thurston 1970; Bowers et al. 2000; Zhang et al. 2008). In addition, the floral MCTs may be functionally different from the vegetative MCTs, producing a product that is unique and important to its microenvironment.

Relative Amounts of Secretory Products Produced by MCTs of Different Taxa

Our results visually and qualitatively assessed the amounts of products associated with the MCTs from the perennial *G. tomentella* as high compared to those in the wild annual *G. soja* and in the cultivars Raiden and Clark. We quantified the numbers of mature MCTs with visible secretory products on gynoecia of *G. tomentella* and Raiden, and the differences were striking, with 52% of mature *G. tomentella* but only 4% of Raiden MCTs producing a visible product outside the trichome cells. This comparison is of specific interest to us because two

active terminal cells with dense and condensed cytoplasms and basal stalk cells that are less dense and highly vacuolate. Terminal-cell cytoplasms display a secretory product between plasmalemmas and primary cell walls and between primary cell walls and modified cuticle layer on side closest to gynoecium epidermis. No product occurs outside cuticle layer at this stage. *G*, MCT (*G. tomentella*) similar to one shown in *F* and stained with toluidine blue O. Small vacuoles around terminal-cell nuclei are evident, as is secretory product between terminal-cell walls and modified cuticle layer next to gynoecium. *H*, Portion of a terminal-cell cytoplasm (*G. tomentella*) before appearance of secretory products outside the MCT. Arrays of rough endoplasmic reticulum and many Golgi bodies with attached vesicles (arrowheads) are evident, as is a large vacuole filled with speckled material (asterisk). Scale bars = 500 nm in *A*, *C*; 200 μm in *B*; 20 μm in *D*, *E*; 10 μm in *F*, *G*; 2 μm in *H*.

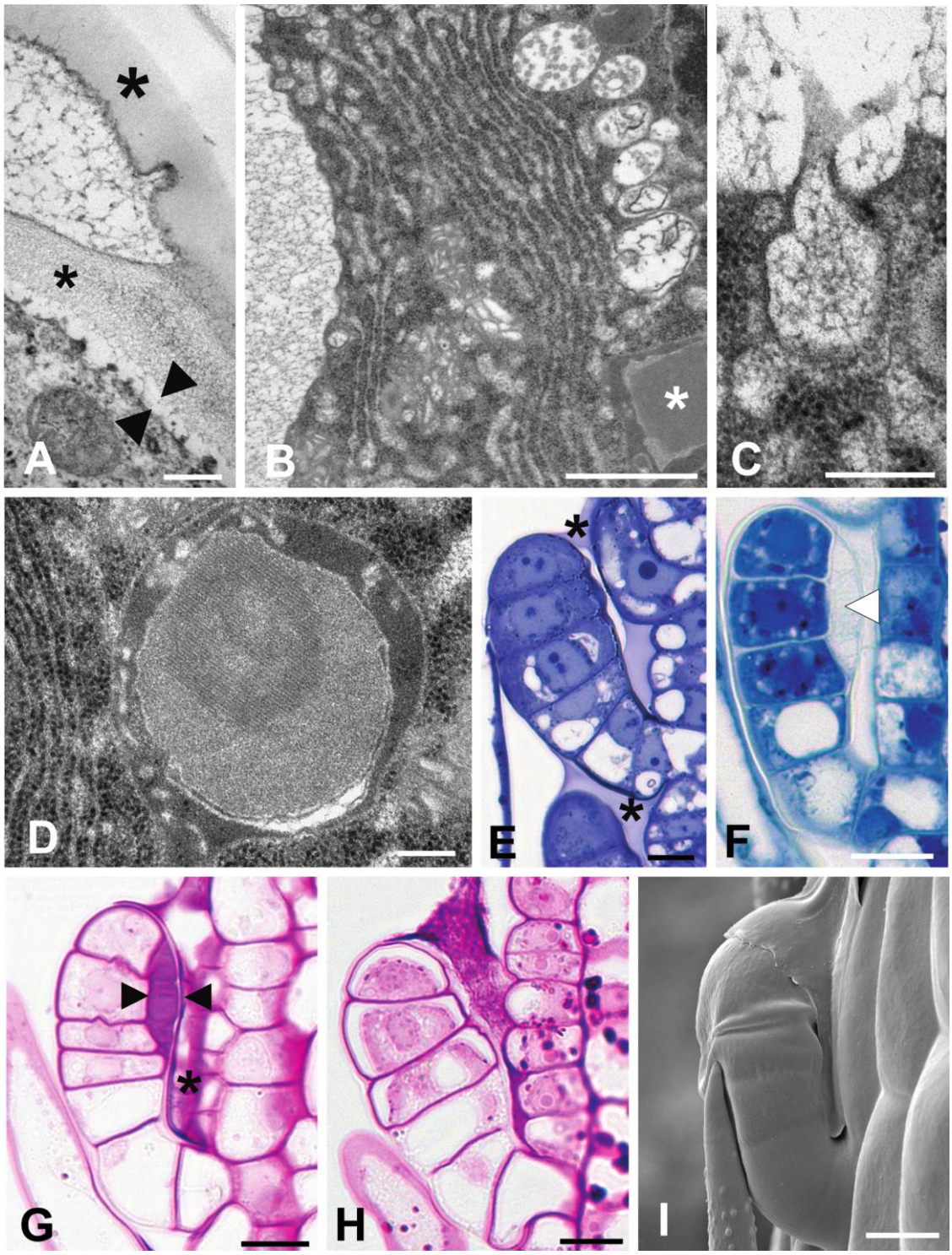

Fig. 5 Late stage of *Glycine* MCT development, viewed with LM, SEM, and TEM, showing high cytoplasmic activity, product secretion, histochemical staining, and deposition of products between MCT and gynoecium epidermis. *A*, Portion of terminal-cell wall (*G. tomentella*) adjacent to epidermis. Fibrous-appearing secretory products occur between plasmalemma (arrowheads) and primary wall (small asterisk), and they are concentrated between primary wall and modified cuticle layer (large asterisk). *B*, Portion of dense, condensed terminal-cell cytoplasm (*G. max* cv. Raiden) showing many ribosomes, parallel arrays of rough endoplasmic reticulum (RER) with enlarged lumens containing fibrous material, Golgi bodies with associated vesicles, and small vacuoles around nucleus containing flocculent material. Plastid with a crystal is shown at lower right (asterisk). *C*, Highly magnified region of a terminal-cell plasmalemma (Raiden) with enlarged portions of endoplasmic reticulum (ER) fused with it. These fused ER extensions contain fibrous material identical to that in extended RER lumens. *D*, Highly magnified terminal-cell

Table 1

PAS-Stained *Glycine tomentella* (G 1300, PI 441.000, Diploid) and *Glycine max* cv. Raiden Gynoecia at Anthesis

	G1300 MCTs			Raiden MCTs		
Section	Total	Mature	Secreting	Total	Mature	Secreting
1	21.5	5	2	32	4.5	0
2	17.5	2	0	23	2.5	0
3	36.5	21	13.5	36.5	1	0
4	21.5	12.5	6	50.5	10.5	0
5	14	6	4.5	76	7	1
6	22.5	17	14	59	2.5	0
7	17	13.5	8.5	23	5.5	0
8	12	6.5	2.5	77	16	1
9	22	15.5	11.5	18.5	4.5	0
10	38	26.5	16	65.5	10.5	.5
11	38	19.5	8.5	74	10	0
12	25.5	14	6	33.5	5.5	1.5
13	9	7	3	30	3.5	0
14	17.5	12.5	5	33	9.5	0
15	27.5	15	6.5	16.5	0	0
16	21	12	7	40.5	4.5	0
17	24	20	11.5	22	3	0
18	35	18	12.5	48	9.5	0
19	19.5	18.5	8.5	36.5	2	0
20	17.5	14.5	6	45.5	6.5	0
21	30.5	17.5	4	4.5	1	0
22	27.5	20	5.5	54.5	4.5	0
23	23.5	4	0	88	15.5	0
24	29.5	23	13	73.5	19.5	1.5
25	29	24.5	15.5	37	5.5	.5
Total	597	365.5	191	1098	164.5	6
Average (%)			<52.3[a]			>3.5[a]

Note. Averages of numbers of total and secreting MCTs.

[a] Percent of mature trichomes displaying secretory products.

other wild perennial species, *G. clandestina* and *G. argyrea*, attract insect pollinators that enhance cross pollination and seed set (Brown et al. 1986; Schoen and Brown 1991), whereas the wild annual *G. soja* is less attractive and Clark is basically nonattractive to insect pollinators. Raiden, however, has been shown to be attractive to insect pollinators in field studies in Texas (Ortiz-Perez et al. 2008*b*), and yet it produces far less product than *G. tomentella*. These observations, along with differences in nectary formation of starch for nectar production (Horner et al. 2007), provide valuable hints and questions about factors that could be intimately involved in insect pollinator attraction. We are incorporating such considerations into current work that includes attempts to collect minute amounts of the pure, uncontaminated (by nectar) MCT secretory products for analysis.

Stalk Cell Walls of MCT

The anatomy of the MCT is linear, and in some ways it is similar to that of many other glandular trichomes already studied. It consists of vacuolated stalk cells with connecting walls that contain prominent plasmodesmata, which we believe facilitate distal movement of nutrients important to the synthesis of secretory product. In addition, the peripheral stalk cell walls and, to some extent, the cross walls stain positively for callose. These results appear to be the first documentation of callose in the stalk cells of glandular trichomes, at least in *Glycine*. Callose is considered an isolating substance that allows only small molecules to pass through it, as seen in many other cellular systems, e.g., the surrounding walls of meiocytes, dyads, tetrads, phloem plates, seeds, wound plugs, leaf and stem hairs, and

plastid (Raiden), with a large central region containing a crystal. *E*, MCTs (*G. tomentella*), stained with toluidine blue O, showing secretory products on the outside next to gynoecium epidermis (asterisks). *F*, MCT (*G. tomentella*) stained with amido black 10B for proteins. Terminal cells stain especially intensely, and secretory products between primary walls and modified cuticle stain lightly. *G*, MCT (*G. tomentella*), stained for non-water-soluble polysaccharides, showing stained primary cell walls and intensely stained products between primary cells walls and outer modified cuticle (arrowheads) toward gynoecium epidermis. Some products (asterisk) occur outside the MCT. *H*, MCT (*G. tomentella*), stained like that in *G* but with more intense staining of secretory products outside of MCT terminal cells and in space next to the gynoecium. *I*, External view of MCT (*G. tomentella*) that displays secretory products between it and the gynoecium. Products cover terminal portion of MCT and extend in both directions. Scale bars = 200 nm in *A*, *C*, *D*; 1 μm in *B*; 10 μm in *E–I*.

pollen tubes (Dong 2005 and references therein; Minic and Jouanin 2006). The stalk cells pass substances through to the distal secretory cells; therefore, the callose seal ensures their passage in one direction without loss, because the entire outer MCT wall cuticle has been modified. This means that only the distal cells leak or secrete molecules that make up the products observed on the outside of the MCT. The callose also may enhance the turgor pressure to move the product through the distal cell walls and modified outer cuticle layer at the secretory stage. All of the gynoecial MCT basal cell walls from the four *Glycine* taxa studied show the presence of significant amounts of callose.

Distal Cell Secretion

One of the main differences noted in field- and greenhouse-grown plants (R. G. Palmer, unpublished observations) is the degree of pod/seed formation among the perennial and the annual taxa. The Australian perennials *G. clandestina* and *G. argyrea* are reported to outcross at a rate of more than 40% (Brown et al. 1986; Schoen and Brown 1991), *G. soja* at about 13%, and *G. max* at less than 1% (Ortiz-Perez et al. 2006). Raiden has been shown to produce high seed set in fields in Texas with appropriate insect pollinators (Ortiz-Perez et al. 2008*a*, 2008*b*).

Even though no products were shown to occur within either the primary walls or the outer, highly modified cuticle around the distal cells, the presence of secretion products is undeniable between the primary walls of the distal cells and the modified cuticle and between the modified cuticle and the gynoecium epidermal layer. These materials seen in LM, SEM, and TEM are found to be associated only with the MCTs and not with the nonsecretory types of trichomes. This leads us to interpret this material as the gynoecium MCT secretory products in all of the taxa studied. The consistency of histochemical staining of these materials within and outside the MCTs also lends support to this interpretation.

The presence and secretion of uncharacterized products from the MCTs is a consistent but variable feature among the taxa and cultivars in this study. The wild perennial MCTs actively secrete, whereas the annuals have low-activity secretion. We believe that the ability of the wild perennial to attract insects for cross pollination could be a critical step in hybridization. Whether the difference in insect pollinator attraction between the wild perennial and the other taxa is related to the amount and composition of the nectar produced by the nectary surrounding the base of the gynoecium or to products produced by the MCTs is not known yet. These results raise the question of why the annual Raiden is relatively more attractive to insects than its companion cultivated lines. There may be dual involvement of both systems: MCT products and nectary quantity and composition.

This study and the ones carried out by Horner et al. (2003) and Healy et al. (2005) clearly point out that the gynoecia of the wild perennials and the wild and cultivated annuals of *Glycine* all display three types of trichomes. However, their number and location vary among the taxa, as does the visibility of the MCTs in comparison to the other two, much larger types of trichomes that cover them. The two taxa with a high degree of insect pollinator attraction and seed set (*G. tomentella* and Raiden; this study) produce such a small amount (<1 μL) of products that collecting them without contamination by nectar and other trichome debris would be a problem. To potentially solve this problem, we are looking at Clark (puberulent near-isogenic line; Healy et al. 2005), which forms many MCTs but relatively few and stunted nonglandular trichomes. We hope that enough MCT secretion products can be collected to assess their chemical nature and determine what role, if any, they play in the process of attracting insect pollinators or protecting the gynoecia and nectaries/nectar from microbial activity.

In assessing the relative degree of secretion and extent of organelle involvement of the perennial species and the annual Raiden, there are some major differences worth noting. In both, the secreting distal cells displayed both arrays of RER with enlarged lumens and vesicle-like sacs containing materials and Golgi bodies that had many vesicles at their secreting faces. In some cases, the endoplasmic-reticulum sacs and Golgi vesicles appeared to contain differentially staining materials. We interpret these two organelles as major factories for synthesis, packaging, and transporting of substances to the boundary of the cells. There, they are secreted to the outside not by wall cracks or pores but apparently by passage through the altered primary-wall matrix and the highly modified cuticle. The histochemical results suggest that these organelles are involved in trafficking synthesized proteins and non-water-soluble polysaccharides and, possibly, other unidentified substances lost during processing for microscopy.

When considering the relatively greater attractiveness to insect pollinators of the perennial *G. tomentella* and the annual Raiden, in comparison to most other *Glycine* wild perennial and annual taxa, it is compelling to understand what the secreted products are and what functional role, if any, they may play during this period in flower and early pod development. Specific identification of the products was outside the scope of this study. However, elucidation of the composition of the products will provide a greater dimension to glandular trichomes, in general, and specifically to those found associated with the vegetative and reproductive organs in the genus *Glycine*.

Acknowledgments

This study is a joint contribution of the USDA-ARS CICGR Unit (R. G. Palmer), USDA Service Center Agency Current Research Information System project 3625-21000-029-01S (H. T. Horner), and the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa 50011, Project 4403 (R. G. Palmer). The microscopic work was carried out in the Microscopy and NanoImaging Facility. C. Van Allen helped with materials for the callose study. We thank Drs. R. Fujita, M. Ohara, K. Okazaki, and Y. Shimamoto for *G. soja* seed of the Ookawa-bashi population. We also thank Drs. Coralie Lashbrook and Nels R. Lersten for their helpful comments while reviewing the manuscript. The mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the USDA or Iowa State University and does not imply its approval to the exclusion of other products that may be suitable.

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